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# Extended Alternating-Temperature Cold Acclimation and Culture Duration Improve Pear Shoot Cryopreservation

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Meristems of many pear genotypes can be successfully cryopreserved following 1 week of cold acclimation, but an equal number do not survive the process or have very little regrowth. This study compared commonly used cold acclimation protocols to determine whether the cold acclimation technique used affected the cold hardiness of shoots or the regrowth of cryopreserved meristems. *In vitro*-grown pear (*Pyrus* L.) shoots were cold acclimated for up to 16 weeks, then either the shoot tips were tested for cold hardiness or the meristems were cryopreserved by controlled freezing. Cold acclimation consisted of alternating temperatures (22°C with light/−1°C darkness with various photo- and thermoperiods) or a constant temperature (4°C with an 8-h photoperiod or darkness). Compared with nonacclimated controls, both alternating- and constant-temperature acclimation significantly improved postcryopreservation regrowth of *P. cordata* Desv. and *P. pashia* Buch. -Ham. ex D. Don meristems. Alternating-temperature acclimation combined with either an 8-h photoperiod or darkness was significantly better than constant-temperature acclimation. Alternating-temperature shoot acclimation for 2 to 5 weeks significantly increased postcryopreservation meristem regrowth, and recovery remained high for up to 15 weeks acclimation. Postcryopreservation meristem regrowth increased with 1 to 5 weeks of constant-temperature acclimation and then declined with longer acclimation. Shoot cold hardiness varied with the acclimation procedure. The $LT_{50}$ of shoots acclimated for 10 weeks with alternating temperatures was −25°C; that with constant temperature was −14.7°C; and that of the nonacclimated control was −10°C. Less frequent transfer of cultures also improved acclimation of shoots. Shoots grown without transfer to fresh medium for 6–12 weeks had higher postcryopreservation recovery with shorter periods of acclimation than shoots with a 3-week transfer cycle. 

*Key Words: Pyrus;* germplasm; meristems; cryopreservation; cold hardiness; photoperiod; osmotic potential; low temperature.

Pear is an important temperate tree-fruit crop throughout the world. Nearly 1500 genotypes are available in the NCGR world pear collection in Corvallis, Oregon, U.S.A. (U. S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository). Field collections are the most common way to preserve tree-fruit germplasm, but can be costly due to the need for land and constant maintenance (16). In addition, the susceptibility of the plants to insects, diseases, and environmental stress is a major constraint to long-term field preservation of these genetic resources. Cryopreservation is considered an ideal method for long-term base storage of germplasm in a relatively small space, free from biotic/abiotic stresses, and amenable to rapid multiplication on demand (41). In the past two decades cryopreservation techniques developed for plant cells, callus, and organs include controlled freezing, vitrification, and alginate encapsulation–dehydration (2). These techniques are available for several *Pyrus* genotypes (10, 22, 27).

A standard controlled-freezing protocol used to screen 60 pear genotypes was successful for about half of the species and cultivars tested (28). This protocol applied 1 week of alternating-temperature cold acclimation. *Pyrus cordata* Desv. and *P. pashia* Buch. -Ham. ex D. Don are two low-chilling pear species that respond poorly to the standard controlled-freezing

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technique. Improvements are needed to develop cryopreservation protocols suitable for the diverse pear germplasm available in the world pear collection.

Growth and nutritional conditions are very important for the development of freezing tolerance in field-grown plants (40). Freezing tolerance is induced by low, above freezing temperatures (35). Most hardy plants, such as woody perennials, cannot tolerate −3°C during the growing season; however, when fully cold acclimated in winter, they tolerate temperatures as low as −196°C (42). Low temperatures, short photoperiod, drought, or abscisic acid (ABA) treatment may influence cold acclimation and thus the freezing tolerance of plants (21, 23, 35, 38). Cold acclimation is associated with many physiological and biochemical alterations, including membrane alterations, changes in protein composition, increases in sugar content, changes in plant hormone concentrations, and alterations in gene expression (5, 8, 14, 20, 32, 35). Cold acclimation and increased freezing tolerance of *in vitro*-grown shoots and meristems are not well understood.

Dehydration techniques and plant dehydration tolerance are two of the most important factors in cryopreservation pretreatments and cryoprotectant regimes. Increasing osmolarity of pretreatment solutions causes cell dehydration and reduction of freezable water. Sucrose and other disaccharides used to dehydrate tissues have interstitial osmotic effects and enter the cells to stabilize proteins and membranes during desiccation (11, 35). Metabolic changes caused by desiccation and low temperature are similar and may be the result of similar mechanisms (21).

Cold acclimation is used as a pretreatment for cryopreservation of many *in vitro*-grown plants. Conditions used for cold acclimation include constant low temperature with short photoperiod (9, 10, 22), total darkness (4), or alternating temperatures with short photoperiod (26, 27, 29). There are no reports directly comparing the effects of these different cold acclimation conditions.

This study compared the effects of cold acclimation with alternating and constant temperatures, several photoperiods or darkness, and the length of cold acclimation on meristem regrowth of pear shoot tips following cryopreservation. In addition, culture duration and plant water content were studied relative to cold hardiness and cryopreservation.

## MATERIALS AND METHODS

### *Plant Material*

Micropropagated shoots of eight *Pyrus* accessions, *P. koehnei* Schneider (PYR 818.001), *P. communis* L. cvs. Beurre d'Amanlis Panachee (PYR 61.001), Bosc-OP-5 (PYR 1165.001), and Monchallard (PYR 397.001), *P. calleryana* Decne. (PYR 661.001), *P. communis* × *P. pyrifolia* (Burm. F) Nakai cv. Good Christian (PYR250.003), *P. pashia* (PYR 871.001), and *P cordata* (PYR 750.001) from the NCGR collection were cultured in Magenta GA7 boxes (Magenta Corp., Chicago, IL, U.S.A.) on Cheng medium (7) with 4.9 μM $N^6$–benzyladenine (BA), 3% sucrose, 0.35% agar (Bitek agar, Difco, Detroit, MI, U.S.A.), and 0.18% Gelrite (Kelco, San Diego, CA, U.S.A.). The pH was adjusted to 5.2 before autoclaving. Growth room conditions were 25°C with a 16-h photoperiod (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and relative humidity at 40%. Shoots (3 cm) were transferred every 3 weeks, except for those in the culture duration experiment.

### *Response of Genotypes to Cold Acclimation*

Eight genotypes (*P. koehnei, P. pashia, P cordata,* Beurre d'Amanlis Panachee, Bosc-OP-5, Monchallard, *P. calleryana,* and 'Good Christian') were tested to determine the effects of acclimation on regrowth following cryopreservation. Three-week old shoots were acclimated at alternating temperatures 22°C with 8 h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/−1°C 16 h darkness (AL8) for 1 week. We extended the acclimation to 6 weeks for some of the genotypes that performed poorly with only 1 week at AL8. Finally, we extended the acclimation to 16 weeks for *P. pashia* and *P. cordata* to determine the effect of extended cold acclimation on the re-

growth of meristems following cryopreservation. Cultures were not transferred to new medium during acclimation.

*Extended Culture Effects*

Shoots of *P. pashia* and *P. cordata* were cultured in the growth room for 3 or 12 weeks without transfer and then placed in alternating-temperature acclimation (AL8) for 0–15 weeks. The osmotic potential and water content of leaves and stems of shoots and the medium were measured and the meristem regrowth was compared following cryopreservation.

*Alternating- vs Constant-Temperature Effects*

Six-week old shoots of *P. pashia* and *P. cordata* were cold acclimated for 1 to 16 weeks without transfer. Five treatments of alternating or constant temperatures combined with short or no photoperiod were used: (1) (AL8) 22°C with 8 h light (10 $\mu$mol · $m^{-2}$ · $s^{-1}$)/−1°C 16 h dark; (2) (AD8) 22°C 8 h dark/−1°C 16 h dark; (3) (CL8) constant low temperature (4°C) with 8 h light; (4) (CD) constant low temperature (4°C) dark; (5) control—constant warm temperature (25°C with 16 h light).

*Photo- and Thermoperiod Effects*

The effect of long, neutral, and short temperature and photoperiod cycles was tested on 6-week old *P. pashia* and *P. cordata* shoots. Shoots were acclimated for 5 weeks with (1) (AL16) 22°C 16 h light/−1°C 8 h dark; (2) (AL8) 22°C 8 h light/−1°C 16 h dark; (3) (AD16) 22°C 16 h dark/−1°C 8 h dark; (4) (AD8) 22°C 16 h/−1°C 8 h dark; (5) (AL12) 22°C 12 h light/−1°C 12 h dark; and (6) (AD12) 22°C 12 h/−1°C 12 h dark.

*Cryopreservation Procedure*

After cold acclimation 25 meristems with attached leaf primordia (0.8–1.0 mm) from shoots of each treatment were dissected and precultured on a firmer medium (0.35% agar/0.185 gelrite) with 5% dimethyl sulfoxide ($Me_2SO$) for 48 h under the same cold acclimation conditions as for the parent shoots. The basic controlled-freezing procedure was described by Reed (27). Meristems were transferred to 0.25 ml liquid medium in 1.2-ml plastic cryo-vials (Cryovial, Beloeil, Quebec, Canada) on ice. The cryoprotectant PGD (12), a mixture of 10% each polyethylene glycol (MW 8000), glucose, and $Me_2SO$ in liquid medium, was added dropwise up to 1.2 ml over 30 min. After 30 min equilibration on ice, the meristems were frozen to −40°C at 0.1°C/min in a programmable freezer (Cryomed, Forma Scientific, Mt. Clemens, MI, U.S.A.) and immersed in liquid nitrogen for at least 1 h. Vials were thawed in 45°C water for 1 min and then in 23°C water for 2 min. The cryoprotectant was removed and replaced with liquid medium. Meristems were plated in 24-cell plates with 2 ml medium per cell (Costar, Cambridge, MA, U.S.A.) for recovery. Regrowth data were taken 4 weeks after thawing. Each experiment used 20 meristems per vial for each treatment and 5 meristems for unfrozen controls, with at least three replications per experiment.

*Cold Hardiness Tests*

A piece (11.4 × 11.5 cm) of moist sterile Kimwipes tissue paper (Kimberly-Clark Corp., Roswell, GA, U.S.A.) was folded and placed into each glass test tube (10 × 75 mm) with 10 pear shoots (1–1.5 cm). The samples were cooled in the programmable freezer at 0.1°C/min and nucleated at −2°C. Five tubes per treatment were removed at 2.5°C intervals from 0 to −50°C. Shoots were thawed at 4°C for 3 h and recultured for 4 weeks on Cheng medium in the growth room to determine viability. Viability was expressed as $LT_{50}$, the temperature at which 50% of the shoots were killed.

*Fresh Weight (FW)/Dry Weight (DW)*

The water content of shoots of *P. cordata* was determined as the difference between fresh weight and dry weight. Dry weights were determined after heating the samples in an oven at 95°C for 24 h. Percentage water was calculated as [(FW − DW)/FW] × 100%.

*Osmotic Potential Measurement*

The water potential of the medium and the shoots of *P. cordata* at different culture or cold acclimation durations was measured using a vapor pressure osmometer (5100C; Wescor, Inc., Logan, UT, U.S.A.). The medium was frozen to −20°C overnight and then thawed for 5 min at room temperature (22°C). Aliquots of the medium (0.5 ml) were centrifuged in 1.5-ml microfuge tubes (Fisher Scientific, Pittsburgh, PA, U.S.A.) at 3000 rpm for 5 min. Shoots (1–1.5 cm) were frozen in the microfuge tubes overnight, thawed for 3 min at room temperature, and then pressed with a glass bar to extract the sap. Sap was centrifuged at 3000 rpm for 5 min to obtain a clear supernatant (13). Osmolalities were converted to MPa $\Psi$ by multiplying by 2.48 (According to Van't Hoff's equation: $-\Psi = -RT\Sigma C_j$, where R is the gas constant, T is the temperature in degrees Kelvin, and Cj is the summation of the concentrations of all solutes in the solution). Each treatment had at least three replications ($n = 30$).

*Statistical Analysis*

The results were analyzed by ANOVA and means were separated with Duncan's multiple range test ($P \leq 0.05$) using Statgraphics *plus* (Statistical Graphics Corp. and STSC Inc., Rockville, MD, U.S.A.).

## RESULTS

*The Response of Genotypes to AL8 Acclimation*

Pear genotypes screened with the standard AL8 cold acclimation procedure had significantly improved postcryopreservation meristem regrowth, but the degree of response varied with genotype (Fig. 1A). *P. koehnei,* 'Beurre d'Amanlis Panachee,' 'Monchallard,' and *P. calleryana* cultured for 3 weeks in the growth room and for 1 week in AL8 acclimation produced >75% regrowth, compared to <25% regrowth for control nonacclimated shoots. Bosc-OP-5, *P. pashia,* and 'Good Christian' responded poorly to 1-week acclimation and required 4–6 weeks for good regrowth (Fig. 1B). *P. cordata* meristem recovery remained low with 1–6 weeks cold acclimation (Fig. 1B).

*Extended Culture Effects*

Extended culture without transfer to fresh medium improved regrowth following cryopreservation for all eight genotypes even without acclimation (data not shown). When combined with AL8 acclimation, long-cultured shoots of *P. pashia* and *P. cordata* reached high postcryopreservation recovery in less time (1 week) than 3-week-cultured shoots (5–8 weeks) (Fig. 2). After 12-week culture without transfer, little growth medium was left in the culture box. The shoots stopped growing, and some formed dormant apical buds. In long-cultured shoots some mature leaves abscised and young leaves died but the shoot tips remained alive and developed higher freezing tolerance than the controls.

Water content of *P. cordata* stems and leaves significantly decreased during the extended culture experiment (Fig. 3A). Over the 3- to 12-week culture duration in the growth room, the water content of leaves decreased by 10% and the osmotic potential of leaves became more negative (−1.37 to −2.22 MPa) (Fig. 3B). The water content of stems declined at the same rate as that of leaves. In contrast to the effects of long-term culture, AL8 acclimation resulted in smaller changes in tissue water content (Fig. 4A). The osmotic potential of leaves and stems decreased significantly after 8 or more weeks of AL8-cold acclimation but remained above −2 MPa (Fig. 4B).

*Alternating-vs Constant-Temperature Effects*

For the first 5 weeks of acclimation, all four treatments significantly ($P \leq 0.05$) improved the postcryopreservation regrowth of *P. pashia* and *P. cordata* meristems (Figs. 5A and 5B). The nonacclimated controls had little or no postcryopreservation regrowth. One-week cold acclimation increased regrowth from 0 to 5–40% for all acclimation treatments, but recovery was still very low for these genotypes. After 3 or 5 weeks acclimation, the regrowth of meristems from shoots grown under alternating-

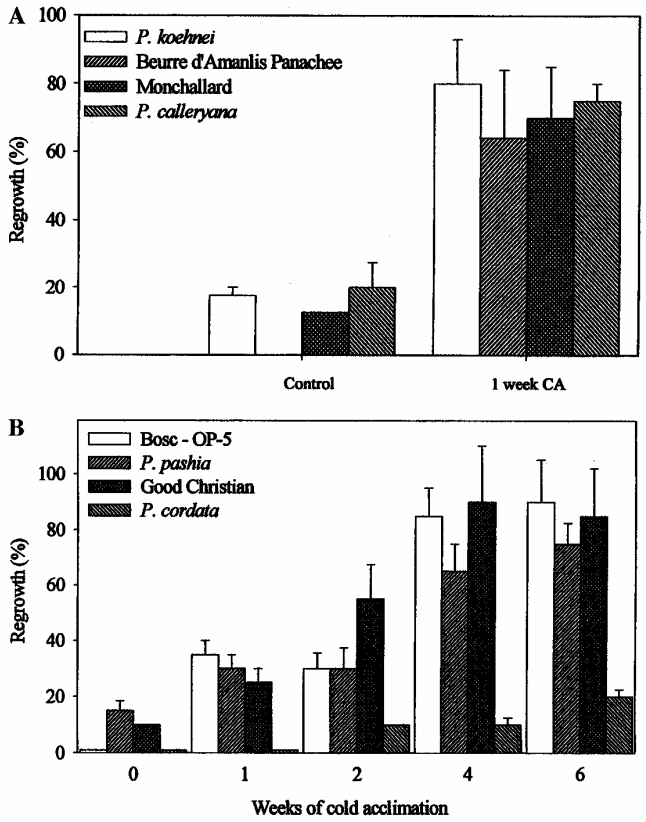


**FIG. 1.** Regrowth of *Pyrus* meristems cryopreserved by controlled freezing. (A) Four pear genotypes grown without cold acclimation (CA) as a control and with 1 week CA. (B) Effects of 0–6 weeks of cold acclimation on four pear genotypes. Cold acclimation conditions: AL8 = 22°C with 8 h light/−1°C 16 h dark. Control conditions 25°C with 16 h light. Results are expressed as means ± SD; $n = 60$.

temperature treatments with either an 8-h photoperiod or darkness was significantly better than that of meristems from constant-temperature-acclimated shoots. At least 3 (*P. pashia*) or 5 weeks (*P. cordata*) of AL8 acclimation were necessary to obtain 80–100% regrowth. Constant-temperature-acclimated meristem recovery generally remained below 40% for *P. cordata* and below 80% for *P. pashia*. Regrowth of meristems with AL8 acclimation remained high throughout the 16-week test, while shoot recovery declined after 5 weeks (*P. cordata*) or 8 weeks (*P. pashia*) with constant-temperature acclimation (Figs. 5A and 5B).

### *Cold Hardiness and Cryopreservation Recovery*

Cold hardiness of both species improved with all acclimation conditions tested (Figs. 5C and 5D). Freezing tolerance of shoots and meristem regrowth following cryopreservation were highly correlated ($R = 0.9025^{**}$) for both *P. pashia* and *P. cordata* (Fig. 5). All four cold acclimation treatments increased shoot cold hardiness. All treatments produced hardiness to −17°C or below by 3 weeks. Constant-temperature acclimation shoot cold hardiness remained steady from 3 to 10 weeks ($LT_{50}$ −17°C).

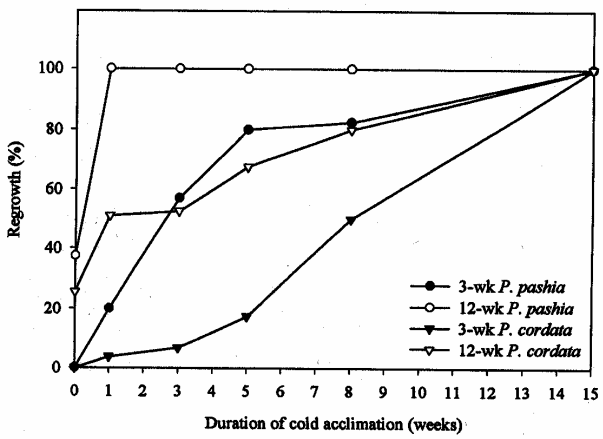


**FIG. 2.** The interaction of culture duration and AL8 cold acclimation on the recovery of *Pyrus pashia* and *P. cordata* meristems following cryopreservation by controlled freezing. Shoots were cultured in the growth room at 25°C for 3 or 12 weeks without transfer to fresh medium before cold acclimation treatment at 22°C with 8 h light/−1°C 16 h dark; $n = 20$.

Shoots grown with AL8 acclimation had the best $LT_{50}$ (−25°C) at 5 weeks, and hardiness remained below −20°C for the remaining 5 weeks.

### *Photo- and Thermoperiod Effects*

Changes in the length of the photoperiod (0, 8, 12, 16 h) or thermoperiod (8, 12, 16 h) did not significantly affect regrowth of the cryopreserved meristems (Table 1). All six alternating-temperature treatments significantly improved the postcryopreservation regrowth of *P. pashia* and *P. cordata* meristems compared to the non-acclimated controls (0% for *P. cordata,* 20% for *P. pashia*); however, there were no significant differences among them. Shoots grown with a 12-h thermoperiod had the highest regrowth for both pear species tested, but it was not significantly better due to the variability observed.

## DISCUSSION

Cold acclimation significantly improved the survival of cryopreserved *Pyrus* meristems, although the required length of acclimation varied among the genotypes. Reed *et al.* (28) divided pear genotypes into three categories based on the amount of AL8 cold acclimation required for moderate to high meristem regrowth following cryopreservation: (1) short, 1 week of cold acclimation, e.g., *P. koehnei* (of 93 pear genotypes tested, 43% belong to this group); (2) medium, 3–7 weeks of cold acclimation, e.g., *P. pashia;* and (3) long, more than 8 weeks of cold acclimation, e.g., *P. cordata.* All the *Pyrus* genotypes tested with the controlled-freezing protocol in our present study fit in these three categories (Fig. 1). When the cold acclimation duration was extended for the four Category 1 genotypes included in Fig. 1A and the three Category 2 genotypes in Fig. 1B, regrowth remained high, indicating that extended cold acclimation was not detrimental (data not shown).

The type of cold acclimation protocol applied to the pear shoots was important for postcryopreservation meristem recovery. Our results showed that alternating-temperature acclimation was much more effective than constant-temperature acclimation for meristem cryopreservation for acclimation periods longer than 1 week (Figs. 5A and 5B). The alternating-temperature conditions used in our studies may have provided strong stimulation of cold-regu-

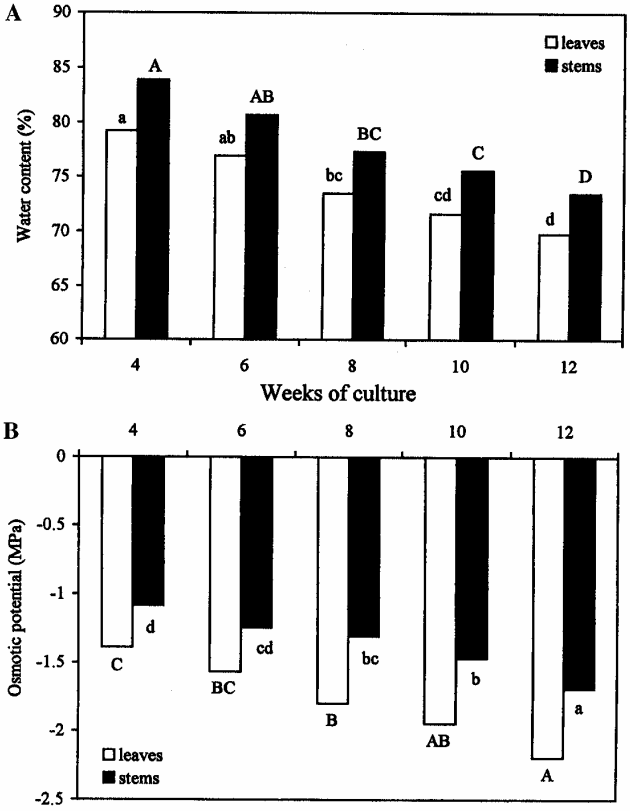


**FIG. 3.** Water content and osmotic potential of leaves and stems of *in vitro*-cultured *Pyrus* shoots. (A) The water content of leaves and stems of *P. cordata* during 12 weeks in culture. (B) The osmotic potential of *P. cordata* stems and leaves after 4–15 weeks in culture. Bars with different letters are significantly different ($P \leq 0.05$), as indicated by capital letters for the leaves and lowercase letters for the stems. Means separation by Duncan's multiple range test.

lated gene expression similar to natural cold acclimation. Constant-temperature treatments initially increased shoot cold hardiness, but after 3 weeks hardiness remained constant at −16°C, while hardiness of shoots in alternating-temperature treatments increased for 5 weeks before stabilizing below −20°C (Figs. 5C and 5D). During the long constant-temperature acclimation we observed that shoots grew slowly or stopped growing for the first several weeks, but exhibited increased growth after 5 weeks (data not shown). Adaptation of shoots to the constant-temperature cold acclimation conditions may have reduced stress and decreased cold-regulated gene expression. Continued growth may also have depleted carbohydrate reserves that serve as cryoprotectants in the cells. Shoots held under alternating-temperature conditions grew little and some formed dormant buds (data not shown). During cold acclimation many changes occur in the cellular structure, physiology, and biochemistry of cells and tissues (6, 14, 37). These changes were evident in the slow growth, compact structure, and dormant bud formation of cold acclimated plants.

Several series of cold-regulated genes in-

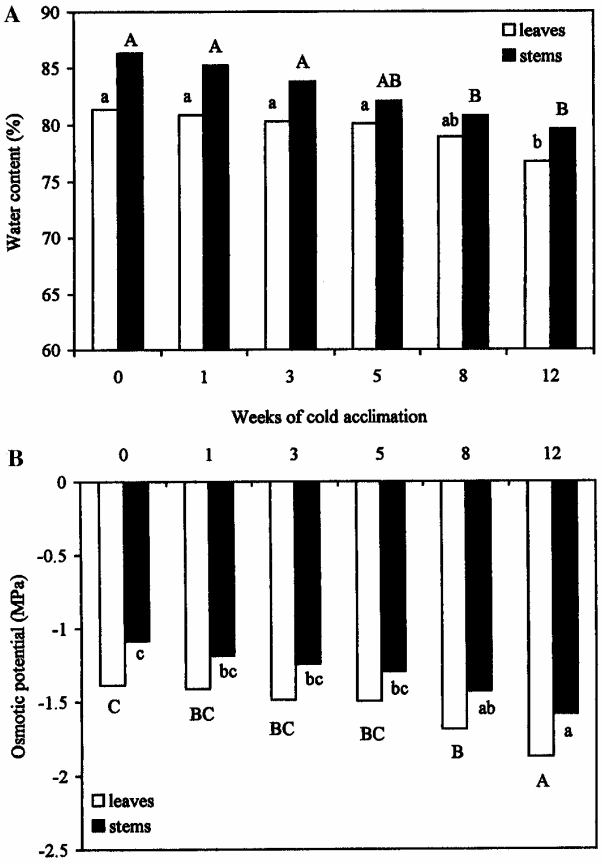


**FIG. 4.** *P. cordata* leaves and stems during 0–12 weeks of AL8 cold acclimation (22°C with 8 h light/−1°C 16 h dark). (A) Water content; (B) osmotic potential. Bars with different letters are significantly different ($P \leq 0.05$), as indicated by capital letters for the leaves and lowercase letters for the stems. Means separation by Duncan's multiple range test.

duced by cold acclimation are now cloned and there is clear evidence that cold-regulated genes play a role in plant cold hardiness (17). Water stress increases freezing tolerance for many plant species and induces some of the same genes as does treatment with ABA and low temperature (20, 42). Water stress is an effective pretreatment for cryopreservation of cell cultures (24, 25). During extended culture the water content of the pear shoots gradually decreased and the osmotic potential became more negative (Fig. 3). The very low osmotic potential of pear leaves and stems following extended culture without transfer to fresh medium indicated that the shoots were in water deficit. This drought stress slightly improved the postcryopreservation regrowth of meristems; however, low-temperature treatment was essential for dramatic increases in regrowth (Fig. 2). Meristems from shoots grown with extended culture not only had increased postcryopreservation regrowth, but also required less cold acclimation for high regrowth than control shoots. Acclimation of hardy species as a result of low-temper-

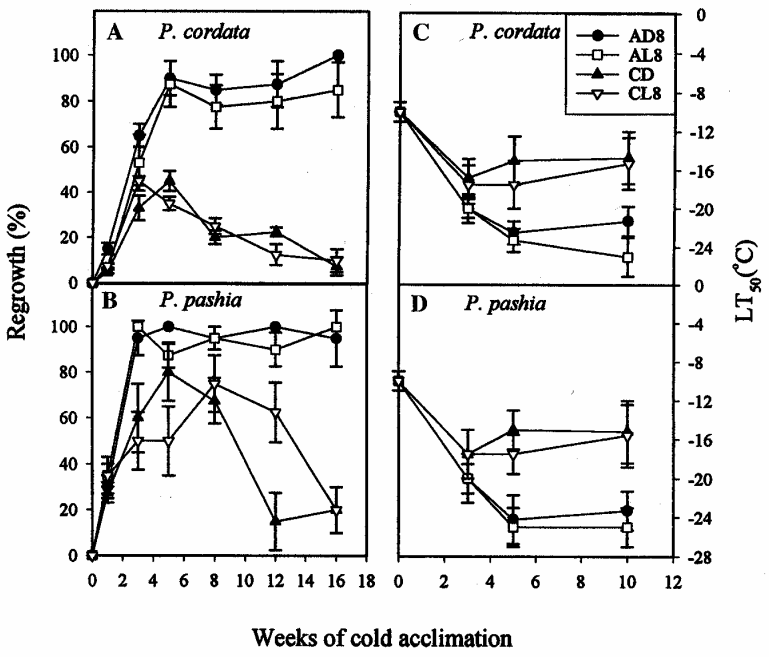


**FIG. 5.** The effect of cold acclimation conditions on cold hardiness of *Pyrus in vitro*-grown shoots and on the recovery of *Pyrus* meristems following cryopreservation. (A) Regrowth of *P. cordata* meristems following cryopreservation. (B) Regrowth of *P. pashia* meristems following cryopreservation. (C) Cold hardiness of *P. cordata* shoots. (D) Cold hardiness of *P. pashia* shoots. (AD8, 22°C 8 h dark/−1°C 16 h dark; AL8, 22°C 8 h light/−1°C 16 h dark; CD, constant low temperature (4°C) dark; CL8, constant low temperature (4°C) with 8 h light. Not shown: Control, constant warm temperature (25°C with 16 h light). Results are expressed as means ± SD. Regrowth $n = 60$; $LT_{50}$ $n = 50$.

ature treatments is associated with early induction and *de novo* synthesis of distinct polypeptides that may stabilize membranes against dehydration stress with resulting increases in freezing tolerance (14, 35). The glass-forming tendency of the cytoplasm of woody plants is also increased with cold acclimation (15, 30).

Reduced water content induced by low temperature is correlated with an increase in freezing tolerance in some species (6, 14); however, this was not the case for pears. Although the water content and osmotic potential of meristems decreased during acclimation (Fig. 4) and were correlated with regrowth following cryopreservation, shoot water content was not an important factor affecting postcryopreservation recovery in our procedure. Meristems of shoots grown in growth-room conditions with extended culture duration had lower water contents than those of cold acclimated shoots (Fig. 3A) but still had less survival following cryopreservation. Desiccation causes increases in abscisic acid in plants, as well as a decrease in protein, RNA, and starch, but an increase in sugar. In *Arabidopsis* cold-regulated genes are both ABA and desiccation responsive (21). During low-temperature cold acclimation many changes occur such as cold-regulated gene expression and the production of dehydrins or dehydrin-like proteins (1, 3, 14) and changes in the plasma membrane structure (32), cellular sugar composition (6), and cell wall components (39). These changes all contribute to cell

TABLE 1
Postcryopreservation Regrowth of *Pyrus* Meristems from Shoots Cold Acclimated for 5 Weeks with Various Thermoperiods and Photoperiods

| Thermoperiod (22/−1°C) | Photoperiod (h) | Regrowth (%) | |
|---|---|---|---|
| | | *P. cordata* | *P. pashia* |
| 8/16 | 8 | 53.7 ± 11.0 | 72.5 ± 17.5 |
| 12/12 | 12 | 67.3 ± 14.1 | 74.6 ± 14.6 |
| 16/8 | 16 | 46.8 ± 19.0 | 63.5 ± 17.8 |
| 8/16 | 0 | 55.0 ± 18.2 | 68.3 ± 7.6 |
| 12/12 | 0 | 72.3 ± 24.4 | 83.4 ± 20.2 |
| 16/8 | 0 | 50.0 ± 5.0 | 58.3 ± 15.3 |

*Note.* Means ± standard deviation; $n = 60$.

cold hardiness and tolerance to freeze-induced dehydration, and decreased water content is likely a result of these processes (8).

Dehydration and intracellular ice crystal formation are the most common causes of plant death during freezing (6, 31, 38). Injury to nonacclimated rye protoplasts resulting from severe cell dehydration is associated with major changes in the ultrastructure of the plasma membrane. Cold acclimation results in alterations in the lipid composition of cellular membranes that increase the membrane stability during freeze-induced dehydration (32, 36). Due to acclimation, solutes increase in the cells and contribute to stabilization of membranes and proteins during dehydration (33). The controlled-freezing process that we used allowed water to move out of cells into the intracellular spaces and crystallize there. We believe that the main cause of meristem death was not from ice formation in the cells, but from freeze-induced dehydration. Freeze-induced dehydration may result in the precipitation of molecules and the denaturation of cellular protein (35), as well as irreversible membrane lesions (33). Shoot water content was well correlated with regrowth and may be important, but the freeze–dehydration tolerance induced by cold acclimation may be more important during the controlled-freezing procedure.

In our study a photoperiod was not required for improved cold acclimation and the length of the photoperiod had no significant effect on cold acclimation or recovery from cryopreservation (Fig. 5 and Table 1). A short photoperiod is required for cold acclimation of many winter annuals, biennials, and perennials in nature (34, 38). In *Arabidopsis* a short photoperiod induces some proteins similar to cold-regulated proteins (18). In poplar trees light is required for photosynthesis to supply sufficient energy for cellular changes or to signal the induction of cold hardiness (19). As with many *in vitro* experiments, sucrose was plentiful in the pear growth medium and may have substituted for any light requirement. Although all cold acclimation conditions tested were very effective for increasing meristem regrowth, the 12/12 h thermoperiod with or without a photoperiod, resulted in the most postcryopreservation regrowth. The 12/12 h thermoperiod regime may provide a good balance of a cold period for inducing necessary cell products and a warm period for continuation of the metabolic activities required for cell survival under adverse conditions.

## CONCLUSIONS

This study showed that the intensity of pear cold hardening varies with the cold acclimation conditions used. Constant and alternating temperatures were equally effective for short acclimation periods; however, for genotypes requiring longer acclimation, the alternating-temperature treatments were clearly more effective. Extended culture alone resulted in slightly increased recovery. Alternating-temperature acclimation combined with extended culture produced deep cold hardiness and high postcryopreservation recovery in pears. These combined techniques allow for the cryopreservation of the complete range of genotypes present in important germplasm collections and the long-term base storage of irreplaceable plant collections.

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